Minimally effective concentration of zoledronic acid to suppress osteoclasts *in vitro*

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Abstract. Zoledronic acid is regarded as the most potent bisphosphonate and is widely used in patients with osteoporosis; however, its side effects, including acute-phase reactions, gastrointestinal complaints, renal dysfunction and bisphosphonate-associated osteonecrosis impair the safety and quality of life of patients. The present study was designed to determine the minimal effective concentration of zoledronic acid through testing the dose-dependent effects of zoledronic acid on osteoclast suppression. A primary culture of bone marrow mononuclear cells obtained from C57 mice (age, 6 weeks) was established and induced to form osteoclasts. The number of multinuclear cells was determined by tartrate-resistant acid phosphatase staining and compared among cultured marrow cells treated with different concentrations of zoledronic acid. Furthermore, the cellular properties, including adhesion, migration and bone resorption, were compared at the minimal effective concentration. At a concentration of $1x10^{-6}$ mol/l, zoledronic acid significantly inhibited the formation of osteoclasts. This inhibitory effect was further enhanced at the concentration of $1x10^{-5}$ mol/l. However, the inhibitory effect of zoledronic acid tapered at the concentration of $1x10^{-4}$ mol/l and there was no further dose-dependent increase. In addition, the concentration of $1x10^{-6}$ mol/l was sufficient to alter cellular functions, including cell adhesion, migration and bone resorption. In conclusion, zoledronic acid was effective in reducing osteoclast formation and suppressing cellular functions. The minimal effective concentration of zoledronic acid *in vitro* was 1 μmol/l. Based on these results, a comparable dosage should be explored in clinical applications.

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Introduction

Bisphosphonates are analogues of pyrophosphate and have been used in the treatment of various clinical conditions (not only osteolytic cancers and bone metastases but also conditions involving osteoclast-mediated bone loss) since the 1960s. As important anti-resorptive agents, bisphosphonates have an important role in metabolic bone diseases and bone metastases (1). Their anti-osteoclastic actions make them important candidates for the adjuvant treatment of giant cell tumors (2). Relative to other bisphosphonates, zoledronic acid has the highest mineral binding affinity, indicating high potency and a long duration of action (3).

As the most potent bisphosphonate, zoledronic acid is widely used in the treatment of patients with osteoporosis, Paget's disease, hypercalcemia, bone metastases and multiple myeloma (3-5). Large doses of bisphosphonates may result in high bioavailability and long, intermittent treatment periods may improve the compliance and persistence of bisphosphonate treatment, particularly for those patients with an overall frail constitution (6). However, the adverse effects of zoledronic acid are not to be underestimated, particularly the renal side effects, which may occur with large dosages. When the dosage of zoledronic acid was increased from 4 to 8 mg, the risk of kidney hypofunction and even kidney failure is significantly increased.

The side effects of zoledronic acid may be grouped into four major categories: Acute-phase reactions, renal side effects, gastrointestinal effects and osteonecrosis of the jaw (7-11). Due to the imposing risks, it is important to know the minimal effective concentration of zoledronic acid. However, this number has rarely been determined and the underlying mechanisms of the anti-osteoclastic actions of zoledronic acid remain elusive (12). The aim of the present study was to analyze the efficacy of zoledronic acid to inhibit osteoclast formation *in vitro*. Specifically, the concentration-dependent influence of zoledronic acid was evaluated in a primary murine osteoclast cell line. The results indicated that zoledronic acid impacted osteoclastogenesis, as well as the adhesive, migratory and bone resorption abilities of the cells. The present study provided a basis for future optimization of zoledronic acid use in the clinic and sheds light on certain previously unreported effects of the drug on osteoclasts.

Materials and methods

Reagents. Zoledronic acid was obtained from Novartis Pharmaceuticals Ltd. (Basel, Switzerland). Macrophage colony stimulating factor (M-CSF) and receptor activator for nuclear factor-κB ligand (RANKL) were obtained from Abcam (Cambridge, UK). Fetal bovine serum (FBS) and tartrate-resistant acid phosphatase (TRAP) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Stock solutions of zoledronic acid were prepared in PBS. Next, 0.1 mol/l NaOH was slowly added dropwise to adjusted the pH to 7.4, and the stock was sterilized by filtration (Millipore syringe-fitted filter, 0.45 μm).

Establishment of primary cell culture. All experiments were approved by the Animal Care and Use Committee of Hebei Medical University (Shijiazhuang, China) and performed by experienced personnel. C57 female mice (age 6 weeks; n=20; weight, 18-20 g) were obtained from the Animal House of Hebei Medical University. Bone marrow cells were obtained from the long limb bone of C57 mice as previously described with minor modifications (13). In brief, mouse bone marrow mononuclear cells were collected by centrifugation (3 x g, 5 min, 37˚C), cultured in α-modified minimal essential medium (α-MEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated at 37˚C in a humidified atmosphere with 5% CO_2 for 72 h. Next, the cells were incubated with a α-MEM supplemented with $3x10^{-5}$ g/l M-CSF, $2x10^{-5}$ g/l murine recombinant RANKL and 10% FBS (13).

After 72 h, the cells were incubated at a density of 10,000 cells/ml with 5 ml α-MEM. Cells were passaged following confluence (80%) and they were separated into six groups. Zoledronic acid was added at a concentration of $1x10^{-4}$, $1x10^{-5}$, $1x10^{-6}$, $1x10^{-7}$ or $1x10^{-8}$ mol/l, while equal volumes of PBS (1 ml) were added to control wells. Zolendronic acid was added during osteoclastogenesis. This process usually takes 48 h, therefore the effect of zolendronic acid was to inhibit osteoclastogenesis. After 24 h, the cell culture medium was replaced with α-MEM containing $4x10^{-5}$ g/l M-CSF, $6x10^{-5}$ g/l murine recombinant RANKL and 10% FBS, followed by incubation for another 120 h (13).

TRAP staining. After 120 h, the cells were fixed with 10% formaldehyde in PBS. Adherent cells were treated with ethanol-acetone at a ratio of 1:1. TRAP staining was subsequently performed as previously described (13). To identify osteoclasts at the end of the culture period, the cells were observed with an inverted microscope (Nikon TE2000S inverted microscope; Nikon Corp., Tokyo, Japan). A scientific research camera and QCapture Pro 7 image and analysis software (QImaging Ltd., Surrey, BC, Canada) were used to capture images and process them. Image-Pro Plus 6.0 software (Media Cybernetic, Rockville, MD, USA) was applied to score and quantify the multinuclear giant cells with positive TRAP staining. Cells with >3 nuclei were regarded as mature osteoclasts).

In vitro adhesion assay of osteoclasts. Bone marrow mononuclear cells were incubated with α-MEM containing $4x10^{-5}$ g/l M-CSF, $6x10^{-5}$ g/l murine recombinant RANKL and 10% FBS. After 120 h, bone marrow mononuclear cells gradually differentiated into osteoclasts. Then the single-cell suspension of osteoclast cells were prepared. These cells were cultured in 96-well plates coated with $4x10^{-5}$ g/l M-CSF and $6x10^{-5}$ g/l murine recombinant RANKL ($5x10^4$ cells/well; Thermo Fisher Scientific, Inc.) and incubated with $1x10^{-6}$ mol/l zoledronic acid or PBS for 1 h. Following the incubation, PBS (pH 7.4) washed the cells three times, removing non-adherent cells, the attached cells then subjected to TRAP staining, and TRAP-positive cells were fixed, observed and counted as previously described (13).

In vitro osteoclast migration assay. First, bone marrow cells were suspended and incubated for 30-40 min. It is known that cells adhere to the wall of the cell culture dish, and that the bone marrow mononuclear cells gradually grow and differentiate into osteoclasts. At the beginning, other cells, including bone marrow stromal cells, were present; however, as the culture time progressed and the media was changed several times, the osteoclasts adhered to the wall of the well more firmly. Following the washing out of other impurities, the majority of the osteoclasts remained as they adhere to the walls firmly. A Transwell system (24-pore plate; 8-μm pore size; Corning-Costar, Corning, NY, USA) was used to analyze and measure osteoclast migration as previously described (13). Osteoclasts ($1x10^4$ cells/chamber) induced by $3x10^{-5}$ g/l M-CSF or $1x10^{-6}$ mol/l zoledronic acid were added to the Transwell system to the experimental chambers, and 200 μl PBS and $3x10^{-5}$ g/l M-CSF were added to the control chambers. All these cells were incubated for 4 h. Then, the cells that had transgressed through the Transwell membrane were observed and quantified.

In vitro resorption of osteoclasts on dentine slice assay. Bone resorption is the characteristic function of osteoclasts (they are also known as bone-resorbing cells). The bone marrow mononuclear cells were incubated in 96-well plates at $5x10^4$ cells/well, bone marrow mononuclear cells gradually differentiated into osteoclasts as specified above. Single-cell suspensions of purified osteoclasts ($1x10^4$ cells) were obtained and seeded on pre-wetted dentine slices (American Laboratory Products Co., Windham, NH, USA) in the presence of $4x10^{-5}$ g/l M-CSF and $6x10^{-5}$ g/l murine recombinant RANKL, followed culture at 37˚C for 16 h. Zoledronic acid ($1x10^{-6}$ mol/l) was then added to the experimental wells, while equal volumes of PBS were added to the control wells. After toluidine blue staining, bone lacuna was observed and measured *in vitro*. Reflective light microscopy was used to observe each bone slice under low-power magnification, and the bone lacuna area was calculated with Image-Pro Plus 6.0 software (Media Cybernetics).

Statistical analysis. Values are expressed as the mean ± standard deviation. SPSS software for Windows (version 19.0; IBM Corp., Armonk, NY, USA) was used for all statistical analyses. The results of the osteoclast formation assay were compared between groups using analysis of variance according to post hoc testing by the Tukey-Kramer method. This method was used to determine the association between various concentrations of zoledronic acid. The results of the osteoclast adhesion assay, migration assay and bone

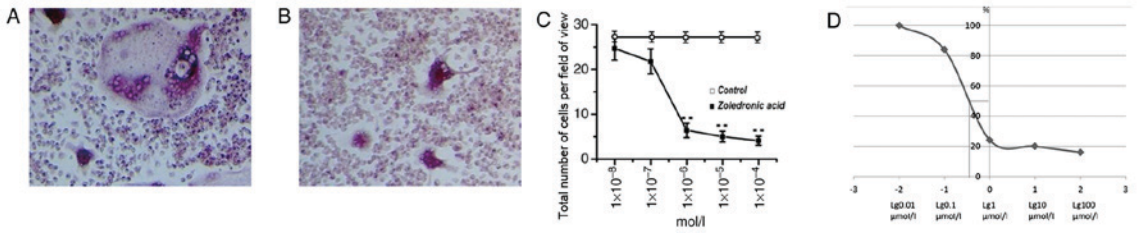

Figure 1. Suppressive effect of zoledronic acid on osteoclast formation. Osteoclast formation was assessed by scoring the total number of multinucleated tartrate-resistant acid phosphatase (+) cells per high-power field. Representative photomicrographs of (A) the control group and (B) the $1x10^{-6}$ mol/l zoledronic acid group (magnification, x400). (C) Quantitative evaluation of the total number of osteoclasts across the groups *in vitro*. (D) S-shaped curve shows the 50% effective concentration of zoledronic acid. **P<0.01 vs. control.

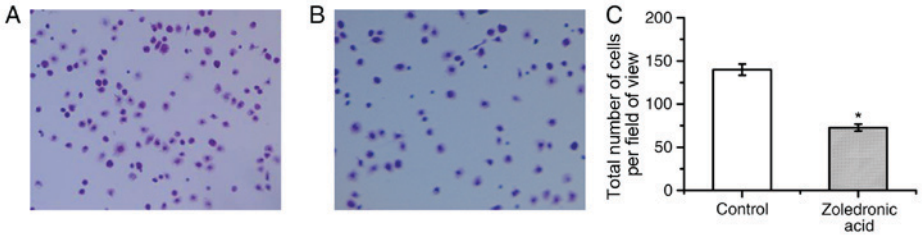

Figure 2. Osteoclast adhesion after treatment with $1x10^{-6}$ mol/l zoledronic acid. Representative photomicrographs demonstrating the adhesion of osteoclast precursors in (A) the control group and (B) the $1x10^{-6}$ mol/l zoledronic acid group (magnification, x100). (C) The amounts of adherent osteoclasts *in vitro* were quantified and compared between the two groups. *P<0.01 vs. control.

resorption assay were statistically analyzed using the Student's t-test. P<0.05 indicated that the difference between groups was statistically significant.

Results

Osteoclast formation declines at $1x10^{-6}$ mol/l of zoledronic acid. Zoledronic acid was effective across all experimental concentrations according to the quantitative evaluation of cells stained with TRAP. Compared with the control group, zoledronic acid directly suppressed osteoclast formation at all concentrations. However, the effectiveness was not obvious at the concentrations of $1x10^{-8}$ and $1x10^{-7}$ mol/l. At the concentration of $1x10^{-6}$ mol/l, the total area of mature mouse osteoclasts was significantly decreased (P<0.01). This inhibitory effect was further enhanced at the concentration of $1x10^{-5}$ mol/l. Furthermore, at the concentration of $1x10^{-4}$ mol/l, the suppressive effect was slightly greater compared with zoledronic acid at $1x10^{-5}$ mol/l. However, no significant suppressive effect was detectable at the concentration of $1x10^{-4}$ mol/l. Importantly, a simple and fitted S-shaped curve was generated and the 50% effective concentration of zoledronic acid was $0.6x10^{-6}$ mol/l (Fig. 1).

Zoledronic acid at $1x10^{-6}$ mol/l exerts a suppressive effect on osteoclast adhesion. Based on the observation of inhibition of osteoclast formation by $1x10^{-6}$ mol/l zoledronic acid, the possible inhibitory effect of this concentration of zoledronic acid on the adhesion ability of osteoclasts was next examined. Attached TRAP-positive cells were fixed and quantitatively evaluated. The number of attached, TRAP-stained cells was 139.7±16.8 cells per high-power field (HPF) in the control group and 72.6±13.1 cells/HPF in the experimental group. Thus, compared with the control group, $1x10^{-6}$ mol/l zoledronic acid (equivalent to pharmacologically administered dose, 2-4 mg) was sufficient to significantly inhibit the adhesion ability of osteoclasts (P<0.01; Fig. 2).

Zoledronic acid at $1x10^{-6}$ mol/l markedly suppresses the migration of osteoclasts. One pre-condition for bone resorption is the migration of osteoclasts. Therefore, the impact of $1x10^{-6}$ mol/l zoledronic acid on the migration ability of osteoclasts was examined. The number of osteoclasts transgressing through a Transwell membrane was determined. The results indicated that in control group 119.6±16.2 cells/HPF were migratory as compared with 36.6±6.1 cells/HPF in the experimental group. This result demonstrated that $1x10^{-6}$ mol/l zoledronic acid was sufficient to significantly decrease the number of migratory cells compared with that in the control group (P<0.01; Fig. 3).

Zoledronic acid ($1x10^{-6}$ mol/l) appreciably suppresses bone resorption of osteoclasts. Osteolytic destruction and bone resorption are universally regarded as basic cellular functions of osteoclasts. Bone resorption of osteoclasts may be observed and measured by the size of Howship's lacuna *in vitro*. In the present study, the demarcation of Howship's lacuna was examined and quantified in the two groups treated with $1x10^{-6}$ mol/l zoledronic acid or vehicle *in vitro*. Microscopically, it was observed that Howship's lacuna in the control group was obviously longer and deeper than that in the experimental group. Specifically, the volume of the bone lacuna was 30.9±6.5 cells/HPF in the control group and 5.1±1.5 cells/HPF in the experimental group. Expressed as percentages, the bone lacuna area was 20.8±3.65% in the control group and 2.12±0.44% in the experimental group. A statistically significant decline in the size of Howship's lacuna was observed after zoledronic acid administration *in vitro* (P<0.01; Fig. 4).

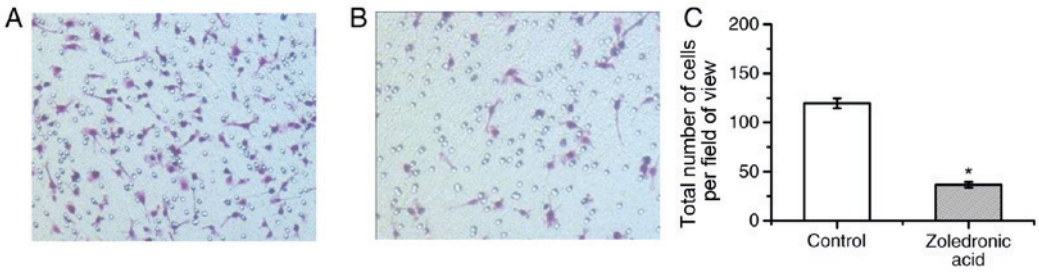

Figure 3. Osteoclast migration was evaluated by a Transwell assay. Representative photomicrographs from 3 independent experiments (magnification, x100). (A) Control group and (B) $1x10^{-6}$ mol/l zoledronic acid group. (C) Quantitative evaluation of migratory osteoclasts in response to treatment with $1x10^{-6}$ mol/l zoledronic acid vs. control. *P<0.01 vs. control.

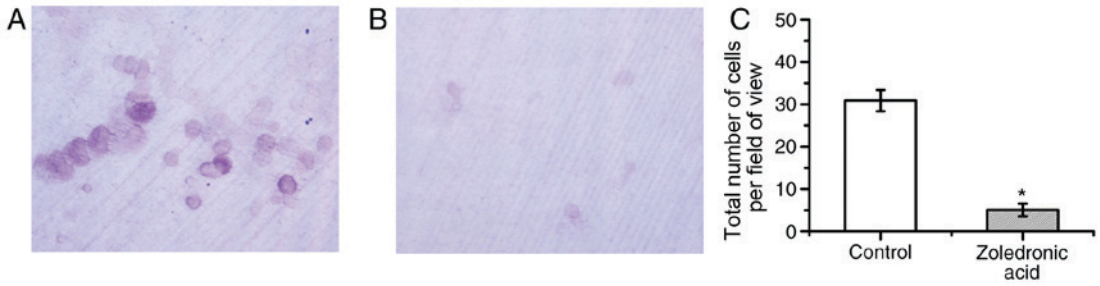

Figure 4. Bone resorption assay. Representative photomicrographs of bone resorption from osteoclast culture in the presence of zoledronic acid (magnification, x400). (A) Control group and (B) $1x10^{-6}$ mol/l zoledronic acid group. (C) Quantitative evaluation of bone resorption in the two groups. *P<0.01 vs. control.

Discussion

Bisphosphonates are non-toxic analogues of pyrophosphate. They share a similar core structure, with one key binding of two molecules (P-C-P), and two side chains or groups, R1 and R2, attached to the central carbon atom. Small changes to the structure of the R2 side chain may alter the anti-resorptive potency by affecting the ability of bisphosphonates to inhibit farnesyl diphosphate synthase (14). The differences in the physicochemical and biological properties of bisphosphonates are due to the differences in the R2 group (14-18). For instance, the presence of nitrogen and its orientation within the R2 side chain may influence the overall potency of various bisphosphonates, and small modifications of the structure of the R2 side chain may afford substantial changes in the anti-resorptive properties of these compounds (19,20).

Due to their anti-osteoclastogenic actions, bisphosphonates have demonstrated efficacy not only in the treatment of osteolytic cancers and bone metastases, but also in other clinical conditions involving osteoclast mediated bone loss (21-23). At present, >10 bisphosphonates have been approved for various clinical applications in various countries, bisphosphonates exhibit differences in the dosages, routes of administration, therapeutic effects and adverse reactions; these differences are meaningful to the patients and clinicians (24-28). The bisphosphonate family is large and a strong structure-activity association of their anti-resorptive potency prevails, likely owing to the fact that each derivation has its own specific mode of action (29).

There are marked differences in the pharmacokinetics of bisphosphonates. Zoledronic acid has the strongest ability to inhibit osteoclastogenesis, followed by alendronate, ibandronate, risedronate and etidronate (14). Alendronate can be taken orally. High mineral binding affinity and intermediate enzyme inhibitory potency are characteristic features of alendronate (29). Therefore, the bone turnover rate is reduced the most with alendronate treatment and its duration of action is the longest (14,29-31). In contrast, due to its moderate mineral binding affinity, risedronate can distribute more widely in the bone (14). The relatively fast onset of action of risedronate is due to its high enzyme potency, although it is lower compared with zoledronic acid (14). Conversely, the enzyme inhibitory potency of ibandronate is higher compared with alendronate (14). Compared with alendronate and risedronate, ibandronate has medium mineral binding affinity (32).

As a highly potent, third-generation nitrogen-containing bisphosphonate, zoledronic acid is the strongest inhibitor of farnesyl pyrophosphate synthase, compared with alendronate, ibandronate, risedronate and etidronate (14). The mineral binding affinity of zoledronic acid is the highest, therefore it has the longest duration of action and the highest potency (33). Zoledronic acid can serve an important role in the loss of osteoclast activity and induction of apoptosis by effectively inhibiting enzymes in the mevalonate signalling pathway, including key regulatory proteins, such as mitochondrial ADP/ATP translocase (34). Moreover, the activity of zoledronic acid on the reduction of osteoclasts and induction of apoptosis is through inhibiting protein prenylation (34). Zoledronic acid is a nitrogen-containing bisphosphonate (35). Clinically, zoledronic acid is approved for the treatment of osteoporosis and cancer patients with osteolysis as it exhibits a high efficacy. In addition, zoledronic acid has a direct effect on cancer cells (9). At the low concentration of $1x10^{-6}$ mol/l, zoledronic

acid can inhibit the invasion of cells (36). In the current study, zoledronic acid was demonstrated to inhibit osteoclast formation, adhesion and migration, and bone resorption at the minimal effective concentration. A study revealed that zoledronic acid can inhibit osteoclast maturation, differentiation and migration, to prevent inflammatory lesion osteolysis (37). Clodronate is a first generation non-nitrogen-containing bisphosphonate and pamidronate is a second generation bisphosphonate nitrogen-containing bisphosphonate (38). Compared with clodronate and pamidronate, zoledronic acid is the most effective drug for inducing apoptosis and inhibiting reabsorption (38). The aforementioned results have important clinical significance.

Zoledronic acid is the most potent bisphosphonate currently known. It is effective in the prevention and treatment of bone-associated conditions. When other bisphosphonates are ineffective, zoledronic acid still relieves cancerous bone pain. The characteristics of bisphosphonates are dose-dependent. However, it was identified that high doses of zoledronic acid did not achieve the desired effect in the treatment of carcinomatous pain (39). Furthermore, the adverse reactions to zoledronic acid were more prominent at high doses. Furthermore, the renal excretion of zoledronic acid is longer than that of bisphosphonates of the same generation (e.g., ibandronic acid). The toxicity to the kidney is relatively high. It remains elusive why high doses of zoledronic acid do not achieve the desired effect. Furthermore, the detailed mechanisms via which zoledronic acid inhibits osteoclasts have remained to be determined. Therefore, the present *in vitro* study examined the effect zoledronic acid on the differentiation of bone marrow cells into osteoclasts, the primary biological characteristics of osteoclasts and the minimal inhibitory concentration. The current study also analyzed and compared the effects of different concentrations of zoledronic acid on the activity of osteoclasts, in order to provide a scientific theoretical basis for clinical treatment. Certain modes of inhibitory action of zoledronic acid on osteoclasts were identified and the lowest effective concentration of zoledronic acid was determined.

In the present study, the influence of zoledronic acid on osteoclastogenesis, adhesion, migration and bone resorption of bone marrow cells was evaluated *in vitro*. Osteoclast formation has been previously reported to be suppressed by high concentrations of zoledronic acid (39,40). Furthermore, a subtoxic concentration (10^{-5} mol/l) of zoledronic acid can prolong the osteoblastic stage span of primary human osteoblasts (40). On the contrary, zoledronic acid at the lowest concentrations, 10^{-6}-10^{-11} mol/l, had no effect on the proliferation of osteoblasts (40). In the present study, the higher concentrations had greater effects, the most pronounced effect of zoledronic acid occurred at a concentration of $1x10^{-4}$ mol/l. However, $1x10^{-6}$ mol/l was the minimum inhibitory concentration. The number of mature mouse osteoclasts at this dose was significantly decreased. As other bisphosphates, zoledronic acid suppressed the formation of osteoclasts in a concentration-dependent manner (13,32). This inhibitory effect was further enhanced at the concentration of $1x10^{-5}$ mol/l. However, the dose-response was not linear, given that the suppressive effectiveness at the concentration of $1x10^{-4}$ mol/l was not significantly higher than that at the precedent concentration. This phenomenon is supported by previous observations describing that the clinical administration of high-dose zoledronic acid did not achieve the desired therapeutic results, particularly in the treatment of osseous metastases of malignant tumors (39). As demonstrated in the current study, the inhibitory effect of zoledronic acid on osteoclasts in culture was not reduced, however no further increases were observed with increasing doses and the dose-response curve was not linear. This characteristic inhibitory effect of zoledronic acid on osteoclasts is supported by clinical studies (40-44). Furthermore, a recent study indicated that in patients with bone metastases due to breast cancer, prostate cancer or multiple myeloma, the use of 4 mg zoledronic acid every 12 weeks (which is a greater administration interval, but required a smaller dose compared with the regime of the current study) did not result in an increased risk of skeletal events compared with the standard dosing interval of every 4 weeks over 2 years (45). In addition, an increased dosage of zoledronic acid did not achieve the desired therapeutic results in another study (42). This is significant particularly due to the known side effects of bisphosphonates, which may be grouped into three major categories: Acute-phase reactions, gastrointestinal effects and renal side effects (41). While certain studies indicate that the anti-angiogenetic effect of bisphosphonates may influence the wound healing process after injury, the side effects of high doses may actually pose more risks than benefits (43-46). This may explain why the clinical administration of high doses of zoledronic acid does not typically achieve the desired efficacy.

The present *in vitro* study provided an appropriate and efficacious concentration of zoledronic acid, and explored its effects on the osteoclastogenesis of bone marrow mononuclear cells and their biological behaviour. As zoledronic acid has been approved for clinical use, the effective and appropriate doses should be established. Several calculations were performed and it was found that $1x10^{-6}$ mol/l zoledronic acid is equivalent to the pharmacologically administered dose of 2-4 mg. In particular, the administration dose (2-4 mg) is required to achieve plasma levels of $1x10^{-6}$ mol/l in an adult person (47). However, the concentration of zoledronic acid in the bone tissue will be higher due to preferential concentration and enrichment of zoledronic acid to bone tissue (47). Through a new experimental model, the appropriate concentration of zoledronic acid in the bone tissue of the patients was calculated to be between $0.4x10^{-6}$ and $4.6x10^{-6}$ mol/l (47). We determined with the effective dose 2-4 mg of zoledronic acid that should be available. When the administration dose comes to 6-8 mg, it demands careful consideration. The effective dose of zoledronic acid was 2-4 mg, which is the appropriate dose in clinical practice; larger doses of 6-8 mg are not recommended. In a future study, an appropriate concentration should be translated into the corresponding dose for clinical administration, to validate its therapeutic effectiveness *in vivo*. The present study also supports the proposition that there is no requirement for large doses of zoledronic acid, due to side effects associated with a high concentration and long durations of treatment. Therefore, the honing in on the appropriate dosage and duration of zoledronic acid treatment is important for reducing the incidence of adverse events in humans. Toward this aim, future studies should be performed to assess the pharmacological effects, at a cytological and pathological basis, of zoledronic acid at

a dose comparable to the minimum effective concentration determined in the present study. This in turn will ensure the optimal use of zoledronic acid and similar bisphosphonates for the treatment of various diseases.

The inhibition of osteoclasts formation and bone resorption by zoledronic acid may have already been performed during the early stages of the development of zoledronic acid as a drug to treat bone diseases (14). In contrast to other studies, the present study aimed to determine the minimally effective concentration of zoledronic acid to suppress osteoclast formation *in vitro*, as an unconventional approach to identify means of administering zoledronic acid in an optimized way without any side effects. Therefore, the present study is pushing back the frontiers and opening doors to reveal why high doses of zoledronic acid did not achieve the desired effect and how zoledronic acid inhibits osteoclasts.

One limitation of the present study is that the dose-dependent effects of zoledronic acid on cell adhesion, migration and bone resorption were not determined. Another limitation is that the *in vitro* experiments cannot imitate the complex drug metabolism and progression of the bone diseases associated with osteoclastogenesis *in vivo*. The present *in vitro* study only explains certain details and features of the pharmacological mechanisms of osteoclast suppression. The effect of zoledronic acid *in vitro* is different from that *in vivo*; therefore, further studies are required to verify the optimal administration schedule and dosing of zoledronic acid *in vivo*. To assess this, clinical trials will be performed.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ and XJ designed the study. YS and HL performed the experiments. LW and CN collected and analyzed the data. PL performed the interpretation of the data and was a major contributor in writing the manuscript. All authors reviewed the initial manuscript and revised it critically for important intellectual content. The final version of the manuscript has been read and approved by all authors, and each author believes that the manuscript represents honest work.

Ethical approval and consent to participate

The design of the present study was in line with scientific and ethical principles. All animal experimental protocols were reviewed and approved by the Animal Care and Use Committee of Hebei Medical University (Shijiazhuang, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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